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The insulin-like growth factor (IGF) system has been shown to play an important role in the progression of a number of cancers, including breast cancer. IGF-1 binds with high affinity to a family of proteins known as the IGF binding proteins (IGFBPs), which act as natural inhibitors of IGF-1 through sequestration from the IGF-1 receptor. The purpose of this proposal is the development of IGF antagonists based on the structure of the IGFBPs. Knowledge of the mechanism of IGFBP action is currently unclear, therefore we have taken a photoaffinity labeling approach to identify the IGF binding domains on the IGFBPs. Previous data collected in the lab indicate that the carboxy terminus of IGFBP-2 is involved in high affinity binding to IGF-1; these experiments are being repeated with IGFBP-3 and IGFBP-5 to extend these findings. Current photoaffinity labeling data indicate that the midregion of the IGFBP-3 protein is involved in IGF-1 binding. This information will be used in the rational design of therapeutic IGF antagonists for use in the treatment of breast and other cancers.

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Caroline Evangelista Harbeson

Introduction

The work detailed in this report involves research into the insulin-like growth factor (IGF) system, which has been shown to play a role in the progression of breast cancer via activation of the IGF-1 receptor. IGF-1 also binds with high affinity to a family of proteins called the IGF binding proteins (IGFBPs), which serve as natural IGF-1 inhibitors through sequestration of IGF-1 from its receptor. Currently no therapeutic IGF antagonists exist. The purpose of this project is the development of novel therapeutic IGF antagonists based on the structure of the IGFBPs. A photoaffinity labeling approach is being used to identify the IGF binding domains on the IGFBPs. This information will be used in the rational design of therapeutic IGF-1 antagonists for use in the treatment of breast cancer.

Body

Specific Aim 1 as outlined in the Statement of Work involves expression of IGFBP-3 and IGFBP-5 in bacterial and mammalian expression systems. The cDNA for IGFBP-5 has been successfully cloned into an inducible bacterial expression system. The protein is produced by the bacteria, which are then lysed to collect the cell contents that contain the IGFBP. The bacteria makes the IGFBP in an unfolded state that must be treated with reducing agents (such as urea) and then carefully refolded to obtain the native, disulfide-bond containing conformation of the protein. The current yield from an average preparation from bacteria (500 ml culture) is low, however, in microgram quantities instead of the expected milligram amounts even in the presence of the inducing agent isopropyl β-D-1-thiogalactopyranoside (IPTG). The purification procedure is currently being optimized to include clones of bacteria that have demonstrated higher expression of the binding protein. The cDNA for IGFBP-3 has been successfully transfected into Chinese hamster ovary (CHO) cells for mammalian expression, which secrete the protein in its native folded and glycosylated form. Clones that express IGFBP-3 are currently being isolated and analyzed to determine protein yield. Several clones were previously isolated that expressed IGFBP-3 but these cells were lost due to contamination problems in the tissue culture facility. New clones are currently being analyzed and will be used to collect significant amounts of IGFBP-3. This expression system involves using methotrexate to induce amplification of the IGFBP-3 DNA incorporated into the mammalian chromosome. Increasing concentrations of methotrexate in the feeding media are being used to amplify the amount of protein being produced by the CHO cells. This has been a slow process because it takes time for the cells to adapt to the presence of methotrexate in their media, which initially kills a large percentage of the cells. A bacterial expression system has not been developed for IGFBP-3 as yet, and IGFBP-5 cDNA has not yet been transfected into CHO cells for mammalian expression. In the meantime, we have been purchasing recombinant IGFBP-3 in order to perform our photoaffinity labeling experiments. This form of IGFBP-3 has been prepared in bacteria and is therefore non-glycosylated. Bacterially expressed IGFBP-5 is not currently commercially available; therefore the experiments outlined using IGFBP-5 have not been performed.

Photoaffinity labeling experiments have been carried out using IGF-1 derivatized at the Gly-1 residue using 4-azidobenzoyl-N-hydroxysuccinimide ester (HSAB). This photoprobe, referred to as abGly1-IGF1, has been

crosslinked to IGFBP-3 as outlined in Specific Aim 1. Mass spectrometric analysis of the reduced and alkylated and trypsin digested photolabeled complex reveal photoincorporation of the IGF photoprobe into the carboxyl terminus of the IGFBP-3 protein. This result is in accordance with our hypothesis, which predicted that the carboxy terminus of IGFBP-3 would be identified to be involved in IGF binding. Specifically, we have shown that residues 188-206 of IGFBP-3 contact the IGFBP-binding domain on IGF-1. Figure 1 shows the MALDI-TOF-MS spectrum of the fragment containing the possible crosslinked fragments with a mass of 4830.69 Da (Fig. 1). MS/MS analysis of this sample was unable to confirm the crosslinking of this fragment of IGFBP-3 to IGF-I.

Specific Aim 2 of the Statement of Work involved identifying a potential site of interaction between the IGFBPs and the IGF-1 receptor binding domain on IGF-1 using crosslinking reagents attached to the Lys-27 residue of IGF-1, a residue that is located near the IGF-1 receptor binding domain. This photoprobe is referred to as abLys27-IGF1. We report that the abLys27-IGF1 photoprobe contacts IGFBP-2 within the midregion of the protein. Specifically, it contacts IGFBP-2 within residues 155-158, as shown in Figure 2. These findings are unexpected based on our hypothesis, which predicted that IGFBP-2 would contact the IGF-1R binding domain within its amino-terminal region. However, MS/MS analysis of the sample indicated that the mass was in fact a tryptic fragment of IGFBP-2. Figure 3 shows the tandem MS/MS of the crosslinked sample, which is identical to a fragment observed from a sample containing tryptic fragments of only IGFBP-2 (Figure 4).

Work published recently in our laboratory demonstrated that Lys27 does in fact contact IGF-I during binding. Robinson *et al.* demonstrated that IGF-I biotinylated at Lys27 retained high affinity IGFBP binding, but poorly labeled the IGFBPs in ligand blot or BIAcore analysis, due to the sequestration of the biotin. These results indicated that Lys27 makes contact with IGFBP-2 and IGFBP-3 (Ref. 1). Since Lys-27 is not directly involved in IGF-1 binding to the IGF-1R, we believe that these findings are demonstrating that there is an important conformational change occurring when IGF-1 interacts with IGFBP-2. We believe that the carboxy-terminus of IGFBP-2 binds with high affinity to the IGFBP-binding domain on IGF-1, and that this allows IGFBP-2 to contact the IGF-1R binding domain, which is located on the opposite face of the IGF-1 molecule from the IGFBP-binding domain. This suggests that IGFBP-2 physically blocks IGF-1 from binding to the IGF-1R and thus providing a role for IGFBP-2 in inhibiting IGF-1 stimulated IGF-1R activation.

Specific Aim 3 of the proposed Statement of Work was to screen random peptide libraries using the phage display technique for identification of amino acid sequences that interact with IGF-1 at the IGFBP binding domain. This work has not been completed. Recently, Deshayes *et al.* described peptide antagonists of IGF-1 function identified by high-throughput phage display. These antagonists are all members of a family of peptides (F1) that possess a distinct highly conserved motiff, CX_9C. One particular peptide was identified that antagonized the interaction of IGF-1 with the IGFBPs and cell-surface receptors at low micromolar concentrations (Ref. 2).

Key Research Accomplishments

- IGFBP-5 has been expressed in an inducible bacterial expression system (*E. coli*) and the purification procedure is currently being optimized.
- IGFBP-3 has been expressed in a mammalian expression system (CHO cells) and the system is currently being optimized.
- Photoaffinity labeling of IGFBP-3 using abGly1-IGF1 identified the carboxy-terminus of IGFBP-3 as being involved in IGF binding.
- Photoaffinity labeling of IGFBP-2 using abLys27-IGF1 identfied a contact site within the midregion of IGFBP-2, suggesting that IGFBP-2 may contact the IGF-1R binding domain on IGF-1, thus inhibiting IGF-1 activation of the IGF-1R.

Reportable Outcomes

1. The work described in this report has been presented at the 86th annual meeting of the Endocrine Society, of which I am a fellow/student member. The data was presented in poster format on June 18, 2002 in New Orleans, LA. This poster was awarded a Travel Grant Award for outstanding research by the Student Affairs Committee of the Endocrine Society. (Ref. 3)
2. The work described in this report has been presented at Student Research Day 2003 at the Medical University of SC on November 3, 2003 in poster format. This poster was awarded a 2nd place prize in the session. (Ref. 4)

Training Accomplishments and Future Directions

- The student is currently writing a thesis detailing the work summarized in this report.
- Future directions include reiteration of these experiments using other IGF binding proteins, including IGFBP-4, IGFBP-5 and IGFBP-6, using both photoprobes (abGly1-IGF-I and abLys27-IGF-I).

Conclusions

In conclusion, several points of Specific Aim 1 of the proposed Statement of Work have been completed. IGFBP-3 has been expressed in a mammalian system, and IGFBP-5 has been expressed in a bacterial system, and both systems are currently being adjusted to promote optimal protein yield. Current photoaffinity labeling experiments with IGFBP-3 have identified a key region of the carboxy-terminus of the protein as being involved in IGF-1 binding. This is an important finding because a number of studies in the literature have implicated either the carboxy or amino terminus as containing the binding site for IGF-1. This data corroborates data in the literature as well as data obtained in our laboratory that indicates that the carboxy-terminus is important in IGF-1 binding. This finding is different from what we found with IGFBP-2 (Ref. 5), so we can conclude that the interactions of the IGFBPs are unique to each protein in the sense that each binding protein interacts with IGF-1 using a different mechanism. Work on Specific Aim 2 has been completed using the photoprobe abLys27-IGF1. We reported that this IGF-1 photoprobe contacts IGFBP-2 within the midregion of the protein, however this data was not confirmed by tandem MS/MS analysis. Other work in our laboratory suggests that IGFBP-2 utilizes a steric-hindrance mechanism in blocking IGF-1 activation of the IGF-1R. Work on Specific Aim 3 has not been completed. The information gained from these studies will aid in the development of novel IGF antagonists based on the structure and function of the IGFBPs.

References

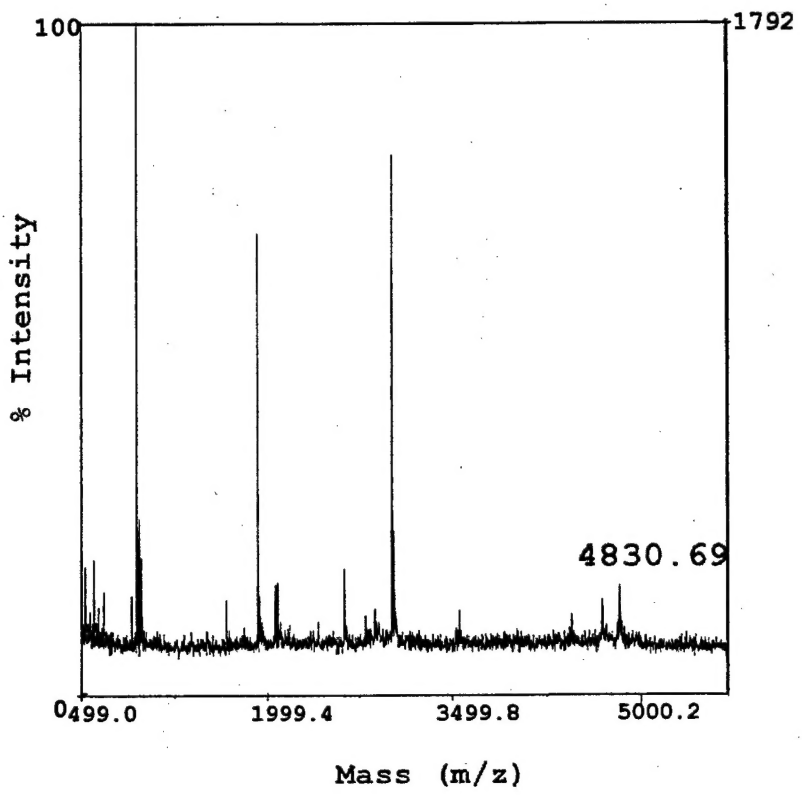
1. Robinson, SA, and Rosenzweig, SA (2004). Synthesis and characterization of biotinylated forms of insulin-like growth factor-1: Topographical evaluation of the IGF-1/IGFBP-2 and IGFBP-3 interface. *Biochem* **43**: 11533-11545.

2. Deshayes, K, Schaffer, ML, Skelton, NJ, Nakamura, GR, Kadkhodayan, S, and Sidhu, SS (2002). Rapid identification of small binding motifs with high-throughput phage display: Discovery of peptidic antagonists of IGF-1 function. *Chem. Biol.* **9**: 495-505.

3. Harbeson, CE and Rosenzweig, SA (2004). Photoaffinity labeling of the insulin-like growth factor (IGF) binding domain on IGF-binding protein-3. *The Endocrine Society's 86th Annual Meeting, abstract book, New Orleans, LA.* (Poster presentation, Travel Grant Winner).

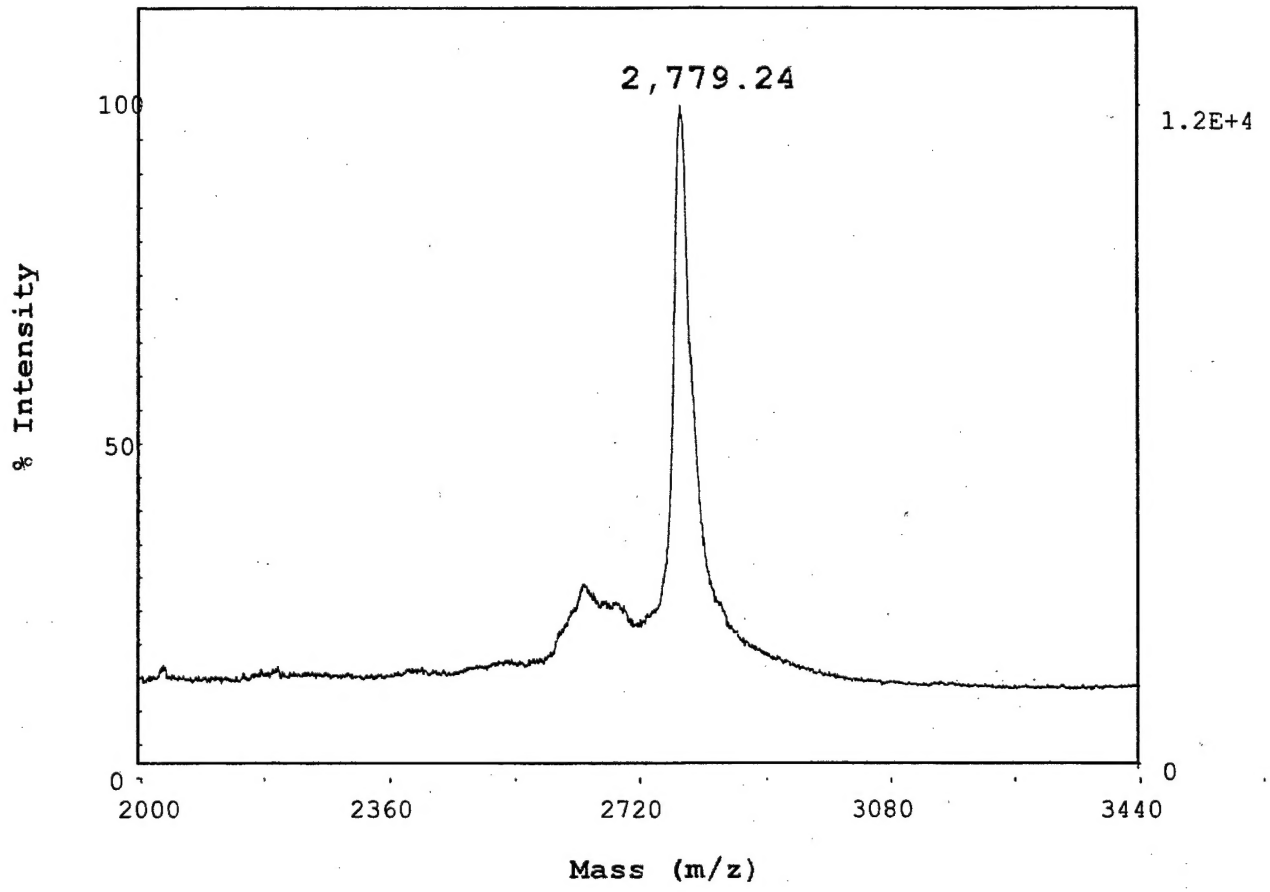
4. Harbeson, CE and Rosenzweig, SA (2003). Photoaffinity labeling of the insulin-like growth factor (IGF) binding domain on IGF-binding protein-3. *Student Research Day, abstract book, November 3, 2003.* (oral presentation).

5. Horney, MJ, Evangelista, CA, and Rosenzweig, SA (2001). Synthesis and characterization of insulin-like growth factor-1 (IGF-1) photoprobes selective for the IGFBPs: Photoaffinity labeling of the IGF-binding domain on IGF-binding protein-2 (IGFBP-2). *J. Biol. Chem.* **276**, 2880-2889.



Observed	Corrected	Predicted	Residues	Sequence
4830.41	2309.34	2312.22	188-206	REMEDTLNHLKFIMSLSPR

Figure 1. Unique masses observed by MALDI-TOF-MS that did not correspond to the mass of any known tryptic fragments of IGFBP-3 were corrected for the mass of the amino-terminal tryptic fragment of IGF-1 (2403 Da) that contains the azidobenzoyl moeity (118 Da). These observed molecular weights listed were found to correspond to residues of IGFBP-3 within the midregion and carboxyl-terminus of IGFBP-3.



Observed	Corrected	Predicted	Residues	Sequence
2779.24	991.4	991.6	111-118	ELAVFREK

Figure 2. Unique masses observed by MALDI-TOF-MS that did not correspond to the mass of any known tryptic fragments of IGFBP-2 were corrected for the mass of the amino-terminal tryptic fragment of IGF-1 (1667 Da) that contains the azidobenzoyl moeity (118 Da). These observed molecular weights listed were found to correspond to residues of IGFBP-2 within the midregion of IGFBP-2.

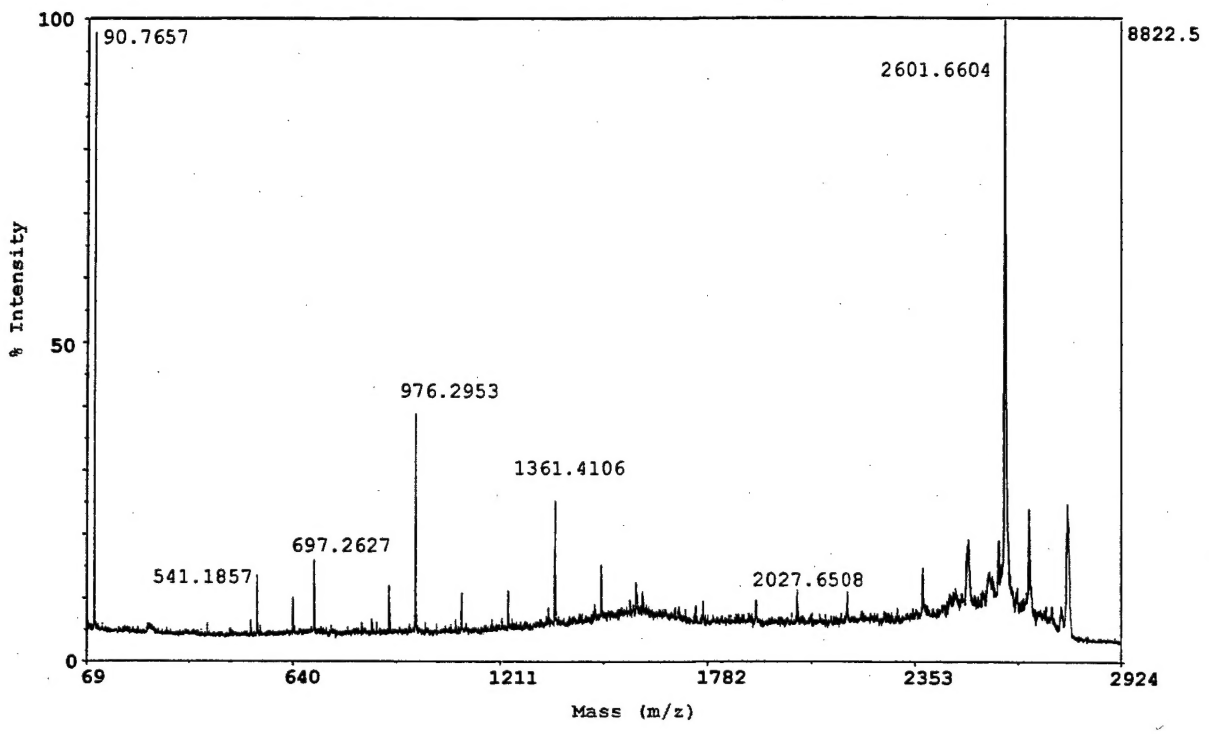


Figure 3. Tandem mass spectrometric analysis of a peak identified by MALDI-TOF-MS (mass 2771.419 Da). This fragment was originally thought to have a unique mass that corresponded to the mass of a fragment of IGFBP-2 (residues 151-158) plus the mass of the tryptic fragment of IGF-1 containing Lys27 (1787 Da) but MS/MS data showed that it is actually a tryptic fragment of IGFPB-2 (shown in Figure 4). This spectrum is identical to the spectrum shown in Figure 4.

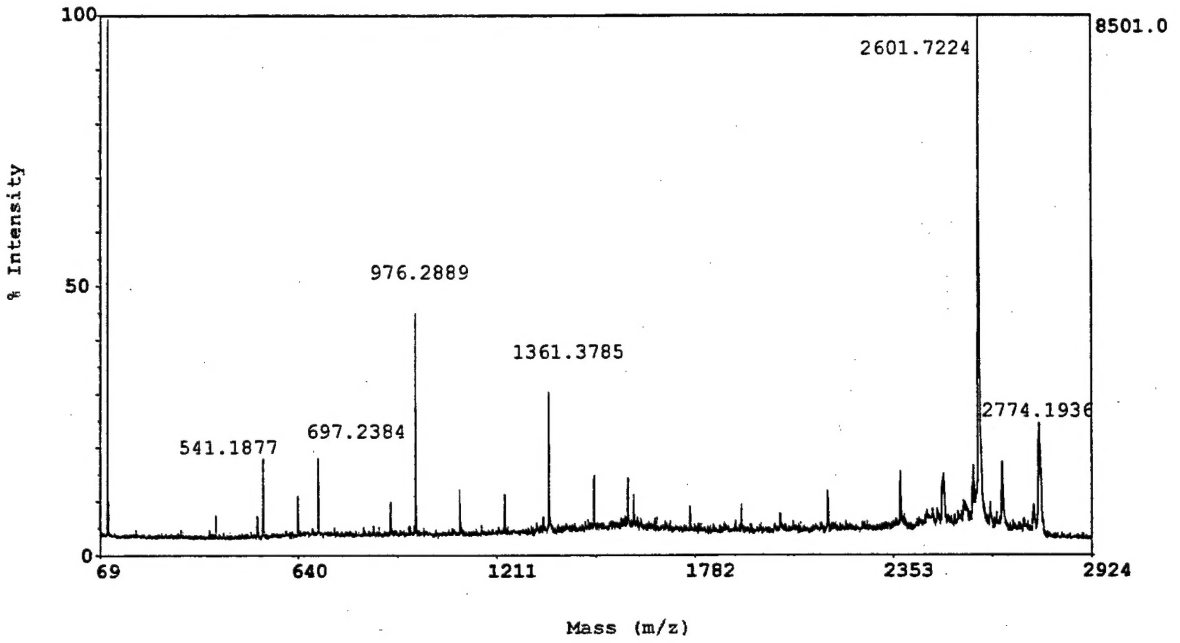


Figure 4. Tandem mass spectrometric analysis of a tryptic fragment of IGFBP-2 (mass 2771.419 Da). This spectrum is identical to the spectrum shown in Figure 3.